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Evaluation of the growth-inhibitory effect of trifluralin analogues on *in vitro* cultured *Babesia bovis* parasites

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ABSTRACT

Bovine babesiosis, caused by *Babesia bovis*, is a global tick borne hemoprotozoan parasite disease characterized by fever, anemia, weight losses and ultimately death. Several babesicidal drugs that have been in use in cattle for years have proven to be only partially effective and the development of alternative chemotherapeutics that are highly specific and have low toxicity against babesiosis is needed. Trifluralin derivatives specifically bind alpha-tubulin in plants and protozoa parasites causing growth inhibition. A set of 12 trifluralin analogues (TFLA) has previously been shown to be inhibitory for the growth of *Leishmania* species. The conservation of several key amino acids involved in the trifluralin binding site of alpha-tubulin among *Leishmania* sp. and *B. bovis* provides rationale for testing these compounds also as babesiacides. The previously tested *Leishmania* inhibitory, TFLA 1–12 minus TFLA 5, in addition to three novel TFLA (termed TFLA 13–15), were tested against *in vitro* cultured *B. bovis* parasites. While all of the TFLA tested in the study showed inhibition of *B. bovis* growth *in vitro* TFLA 7, TFLA 10 and TFLA 13, were the most effective inhibitors with estimated IC_{50} (μM) at 72 h of 8.5 ± 0.3; 9.2 ± 0.2; 8.9 ± 0.7, respectively for the biologically attenuated cloned *B. bovis* Mo7 strain, and 13.6 ± 1.5; 18.7 ± 1.6; 10.6 ± 1.9, respectively for the virulent *B. bovis* T_3Bo strain. The differences found between the two strains were not statistically significant. Importantly, these drugs displayed low levels of toxicity for the host erythrocytes and bovine renal arterial endothelial cells at the doses tested. The demonstrated ability of trifluralin analogues to inhibit *in vitro* growth of *B. bovis* parasites combined with their low toxicity for host cells suggests that these compounds may be further developed as novel alternatives for the treatment of bovine babesiosis.

1. Introduction

Bovine babesiosis is caused by intraerythrocytic protozoan parasites of the genus *Babesia*. *Babesia* parasites are transmitted by Ixodid ticks mainly in tropical and sub-tropical areas worldwide (Boustani and Gelfand, 1996; Homer et al., 2000; AbouLaila et al., 2010). Clinical symptoms can include fever, hemolytic anemia, hemoglobinuria, and severe clinical cases may be followed by sudden death (AbouLaila et al., 2010). However, it is widely recognized that *Babesia bovis* causes higher morbidity and mortality than *Babesia bigemina* (Suarez and Noh, 2011). The only preventive treatment available against bovine babesiosis is based in live-attenuated vaccines that are commonly used in Australia, Argentina, South Africa and Israel (Vial and Gorenflot, 2006), but these vaccines are not licensed for use in the US or in many other countries. Therefore, vaccine use is limited or not widely available in several countries; consequently treatment of cattle with babesiacides plays a central role in the management of this disease.

Babesiacides currently available for treatment of cattle, horses, dogs and other animals include imidocarb dipropionate (Imizol®, Schering-Plough Animal Health) and diminazene aceturate (Berenil®, Intervet, India Pvt. Ltd.). In addition, quinine, clindamycin and atovaquone (Mepron®, Glaxo Welcome) are available for use in humans (Vial and Gorenflot, 2006). Most of these babesicidal drugs have proven to be ineffective owing to problems related to their toxicity, residual effects, and the development of resistant parasites (Bork et al., 2005; Vial and Gorenflot, 2006; Suarez and Noh, 2011). Consequently, the identification of new parasite-spe-

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cific babesicidal drugs that are non-toxic for the hosts remains critical.

Cell microtubules are rapidly assembled and disassembled to create structures that are essential for the development of eukaryotic cells. The current availability of microtubule inhibitor drugs and the occurrence of extensive sequence differences among the proteins involved in these processes among protozoa and the cells of their hosts, suggests that they could be potential drug targets. Compounds that disturb intracellular processes involving microtubules are often used to treat medical conditions, including cancer, gout, and helminth infections (Jordan et al., 1998). Certain microtubule inhibitors, including vinblastine and taxol, display potent activity against parasites by disrupting the microtubular structures of the host erythrocyte, which make them potentially toxic for mammalian cells. In contrast, antimitotic herbicides, namely dinitroanilines (e.g. trifluralin, ethafluralin and oryzalin) that bind tubulin heterodimers and disrupt microtubule in plant cells (Chan and Fong, 1990; Simoncelli et al., 2003; Hashim et al., 2012), do not affect the function of mammalian and fungal tubulins (Chan and Fong, 1990). However, these compounds do inhibit the growth of numerous protozoan parasites such as *Leishmania* species, *Toxoplasma gondii*, *Trypanosoma cruzi* and the *Babesia*, related major human malarial parasite *Plasmodium falciparum*, in culture (Traub-Cseko et al., 2001; Werbovetz, 2002; Fennell et al., 2006; Naughton et al., 2008). Significantly, trifluralin analogues are known not to be lytic or toxic for bovine erythrocytes. Studies performed by Mitra and Sept, 2006 demonstrated that bovine alpha- (α-) tubulin lacks the known consensus binding site for dinitroanilines, and thus displayed only nonspecific and low-affinity interaction with these compounds (Chan and Fong, 1990). Taking together, these observations suggest that dinitroanilines such as trifluralin, while having a great potential for inhibiting protozoan development, might have limited toxicity for the mammal hosts, and thus they could be considered as strong candidates for the development of new alternative and non-host toxic babesiacides.

Trifluralin is effective against the cutaneous form of leishmaniasis when delivered as an ointment (Chan et al., 1993; Esteves et al., 2010). However, its use for controlling other forms of leishmaniasis, namely by parenteral administration, has been limited by its low water solubility and easy sublimation (Mamy et al., 2005). To address these undesirable properties that are characteristic of the dinitroanilines, twelve new trifluralin analogues (TFLA) (termed TFLA 1–12) incorporating chemical modifications in the amine group have been developed, resulting in novel and more effective compounds with increased inhibitory activity on the growth of *Leishmania donovani* and *Leishmania infantum in vitro* (Esteves et al., 2010).

In this study, we first determine that some α-tubulin amino acid residues that are known to participate in the binding site for dinitroanilines in other protozoa parasites are also conserved among *B. bovis*. Based on this observation, we assessed the efficiency of the TFLA 1–12 and the newly synthetized TFLA 13–15 to inhibit the growth of *B. bovis* in *in vitro* cultures. The data collected in this study demonstrates that all previously tested *Leishmania* inhibitory TFLA as well as the newly synthetized TFLA are able to inhibit the growth of *B. bovis in vitro*, apparently without seriously compromising the host erythrocyte cells.

2. Material and methods

2.1. Multiple alignments of α-tubulin sequences

The α-tubulin amino acid sequences from several parasites were aligned using the program ClustalW available at http://www.genome.jp/tools/clustalw/. The GenBank accession no. are: *Leishmania major* (CAJ02502.1, locus tag: LMJF_13_0380); *Trypanosoma brucei gambiense* (CBH08955.1, locus tag: TbgDal_I1360); *P. falciparum* (CAA34101.1); *T. gondii* (AAA30145.1); *B. bovis* (EDO06790.1, locus tag: BBOV_IV004290); *Theileria annulata* (XP_954407.1, locus tag: TA21240) and *Homo sapiens* (NP_116093.1).

2.2. General procedure for TFLA synthesis

All reagents and solvents were of the purest grade available, and generally were used without further treatment. The starting materials, chloralin, imidazole, benzimidazole and sulfanilamide were purchased from Sigma–Aldrich, Europe.

Synthesis of TFLA was performed as previously described (Esteves et al., 2010). Briefly, the triethylamine (1.1 mmol) and the corresponding amine (1.1 mmol) were added simultaneously into a solution of chloralin (1 mmol) in ethanol (5 ml) under nitrogen atmosphere. The mixture was refluxed until complete consumption of amine (0.5–1 h), as monitored by thin layer chromatography (TLC), cooled to room temperature, poured into ice-water and extracted with ethyl acetate. The organic extracts were dried over magnesium sulfate, filtered and concentrated in vacuum to obtain a solid residue. The residues were purified by silica column chromatography and/or recrystallization to afford the products as crystalline solids.

Melting points were determined in a Reichert Thermovar apparatus and are uncorrected. FTIR spectra were recorded on a Perkin-Elmer Spectrum BX v5.3.1 spectrometer. UV/VIS spectra were recorded on a Hitachi U-2800A spectrophotometer. Fourier transform (FT) NMR spectra were run on a BRUKER, AVANCE 400 MHz Ultra Shield spectrometer at Faculty of Sciences of Lisbon University. The chemical shifts are reported in δ (ppm, TMS) and coupling constants in Hz. Electronic Impact (EI) mass spectra (MS) and Electron Spray Ionization (ESI) mass spectra were performed on a Brucker Daltonics Apex-Qe instrument at 300.0 V at CACTI of Vigo University. Microanalyses were performed on a Fisons EA-1108 microanalyzer at CACTI of Vigo University. TLC was performed on silica gel 60 F254 plates with 0.2 mm layer thickness from Macherey-Naguel, and the compounds visualized by illumination under UV light at 254 nm. Column chromatography (CC) was carried out with Macherey Naguel Si gel 60 (230–400 mesh).

2.2.1. 1-(2,6-Dinitro-4-trifluoromethyl-phenyl)-1H-imidazole (TFLA 13)

The general method for synthesis was used with chloralin (0.273 g, 1 mmol), triethylamine (0.15 ml, 1.1 mmol) and 1*H*-imidazole (0.068 g, 1.1 mmol) to obtain the product as light yellow crystals (dichloromethane/hexane, η = 62%). mp 168–169 °C. FTIR (KBr): ν 3430, 3094, 1553, 1318 cm^{-1}. ^1H NMR ($CDCl_3$): δ (ppm) 8.12 (2H, s, H3 and H5), 7.60 (1H, brs, C(NO_2)CNC*H*CH), 7.29 (1H, brs, NC*H*N), 7.05 (1H, brs, CHC*H*N). MS (EI) m/z 302 [M^+] (6), 283 (19), 247 (64), 173 (94), 158 (100). Calc. for $C_{10}H_5F_3N_4O_4$, C, 39.75; H, 1.67; N, 18.54%; found C, 39.83; H, 1.58; N, 18.50%.

2.2.2. 1-(2,6-Dinitro-4-trifluoromethyl-phenyl)-1H-benzo[d]imidazole (TFLA 14)

The general method for synthesis was used with chloralin (0.273 g, 1 mmol), benzimidazole (0.130 g, 1.1 mmol) and triethylamine (0.15 ml, 1.1 mmol) to obtain the product as yellow crystals (diethyl ether/hexane, η = 68%). mp 159–160 °C. FTIR (KBr): ν 3426, 2888, 1551, 1313, 1147 cm^{-1}. ^1H NMR ($CDCl_3$): δ (ppm) 8.60 (2H, s, H3 and H5), 7.99 (1H, s, NC*H*N), 7.90 (1H, d, J = 8, CC*H*CH) 7.40 (1H, t, J = 7 Hz, CHC*H*CH), 7.33 (1H, t, J = 8 Hz, CHC*H*CH), 7.01 (1H, d, J = 8, CC*H*CH) MS (EI) m/z 352 [M^+] (51), 322 (26), 294 (100), 248

(69), 164 (30). Calc. for $C_{14}H_7F_3N_4O_4$, C, 47.74; H, 2.00; N, 15.91%; found C, 47.71; H, 1.90; N, 15.99%.

2.2.3. 4-(2,6-Dinitro-4-(trifluoromethyl)phenylamino)benzenesulfonamide (TFLA 15)

The general method for synthesis was used with chloralin (0.273 g, 1 mmol), sulfanilamide (0.190 g, 1.1 mmol) and triethylamine (0.15 ml, 1.1 mmol) to obtain the product as yellow crystals (acetone/petroleum ether, $\eta = 61\%$). mp 231–233 °C. FTIR (KBr): ν 3345, 3283, 3256, 1535, 1328, 1155 cm^{-1}. ^1H NMR ($CDCl_3$): δ (ppm) 8.68 (2H, s, H3 and H5), 7.83 (2H, d, $J = 9$, C*H*CS), 7.37 (2H, d, $J = 7$ Hz, NHCC*H*). MS (EI) m/z 406 [M^+] (37), 342 (100), 278 (35), 262 (45), 63 (62). Calc. for $C_{13}H_9F_3N_4O_6S$, C, 38.43; H, 2.23; N, 13.79%; found C, 38.46; H, 1.87; N, 13.83%.

2.3. Determination of water solubility

The water solubility values were obtained from the UV spectra recorded for each TFLA. Samples were prepared according to the following general procedure: a suspension of each compound (5 mg) in water (10 ml) was stirred at room temperature for 1 h, filtered and the UV spectrum of the filtrate (3 ml) was recorded and the value of the absorbance at the maximum wavelength (λ_{max}) was obtained. In order to determine the value of the absorptivity at λ_{max}, a standard solution of known concentration was prepared by dissolving the compound (4 mg) in methanol (10 ml) and then diluting this solution in water (5, 12.5 and 25 times) followed by recording the UV spectra for the three dilutions. The value of absorptivity obtained is the average of the absorptivity values calculated for each dilution by applying the Beer law to the UV data. With this value of absorptivity, the concentration of each compound in the water sample was calculated.

2.4. In vitro cultivation of B. bovis parasites

Babesia bovis Texas S_{74}-T_3Bo (derived from S1-T_2Bo, Goff et al., 1998) and Mo7 (Rodriguez et al., 1983) strains were grown in bovine erythrocytes using a continuous micro-aerophilous stationary phase system as described by Levy and Ristic (1980). The cultures were grown in 96-well plate in 10% hematocrit using culture media, HL-1, pH 7.25, supplemented with 40% bovine serum, 0.5 ml of 100× stock solution of antibiotic/antimitotic (Sigma) and 1 mM TAPSO solution (Sigma). Cultures were incubated at 37 °C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

2.5. In vitro B. bovis growth inhibition assay

All 14 TFLA (TFLA 1–4 and 6–15) were evaluated for their chemotherapeutic effect against *B. bovis* in *in vitro* culture. All the compounds were dissolved in DMSO/ethanol 50:50 (v/v) as previously described (Naughton et al., 2008), to a final concentration of 15 mM.

The inhibition assays were performed in a 96-well plate in 10% hematocrit using 180 µl medium per well containing the respective TFLA concentration. Initially, concentrations of 10, 50 and 100 µM of all compounds were tested for their inhibitory effect on *B. bovis* Mo7 strain, and after choosing the three compounds with the best inhibitory effect, concentrations of 10, 20, 40, 60, 80 and 100 µM of TFLA 7, TFLA 10 and TFLA 13 were tested for their inhibitory effect on both Mo7 and T_3Bo strains of *B. bovis*. Four concentrations of imidocarb dipropionate (IMIZOL®, Schering-Plough Animal Health Corp.), 1.75, 5, 10 and 20 µM, were tested on additional cultures. Cultures grown in the absence of drugs and containing only the solvent (DMSO/ethanol) at the highest concentration used in TFLA (0.7%) were tested as controls. The culture medium was replaced every day with 180 µl medium per well containing the respective TFLA or imidocarb dipropionate concentration, and the percentage of parasitized erythrocytes (PPE) was monitored daily by Giemsa-stained erythrocytes smears for 72 h. The average PPE reached at 72 h in the control wells (no solvents or drugs added) was approximately 10%. These experiments were carried out in triplicate for each TFLA concentration. IC_{50} values were calculated for each time point of the *in vitro* culture by extrapolation from the PPE curve as the concentration in which there is a 50% reduction of PPE in the wells treated with drugs compared to the control well.

2.6. Hemolytic activity assay

The percentage of hemolytic activity was estimated as previously described by Naughton et al., 2008. Briefly, the percentage of hemolytic activity of each TFLA at different concentrations was estimated as $(A - A_0/A_{max} - A_0) * 100$, where A_0 is the background hemolysis obtained by incubation of the *B. bovis* Mo7 parasites with culture medium (supernatant) and A_{max} is the 100% hemolysis achieved after incubation in water. The 550 nm measures of absorbance of each supernatant were taken with a reference filter at 620 nm. These experiments were carried out in triplicate for each TFLA concentration.

2.7. Babesia bovis viability assay using 6-carboxyfluorescein diacetate after incubation with TFLA

Babesia bovis Mo7 strain culture was performed in a 96-well plate in 10% hematocrit using 180 µl medium per well containing TFLA 7, TFLA 10 and TFLA 13 at 10 µM, imidocarb dipropionate at 1.75 µM and 5 µM, solvent without drugs (0.7%), or *B. bovis* Mo7 parasites growing in regular culture media (no drugs or solvents). All cultures were grown for 72 h as previously described (Sections 2 and 2.5). The viability assay protocol used was based on Goff et al., 1988. Briefly, 3.5 µl of *B. bovis* infected erythrocytes that were cultured for 72 h were added to 1 ml of citrate saline solution (0.15 M sodium chloride; 0.01 M sodium citrate) and centrifuged at 400×*g* for 5 min. The resulting pellet was washed twice with citrate saline solution, suspended into 1 ml of citrate saline solution containing 2 µl of 6-carboxyfluorescein diacetate (CFDA), and incubated for 20 min at room temperature in the dark. After centrifugation at 400×*g* for 5 min, the pellet was suspended in 15 µl of citrate saline solution, and 2.5 µl was transferred to a glass slide containing 0.8 µl of Slow Fade Antifade reagent (Invitrogen). A cover slip was placed over the top and the slide was examined under fluorescence microscope at 40× amplification. In addition, the percentage of parasitized erythrocytes (PPE) in *B. bovis* infected erythrocytes that were cultured for 72 h was monitored using standard Giemsa-stained slides with an optical microscope. All viability experiments were carried out in triplicate.

2.8. Bovine renal arterial endothelial cells viability after incubation with TFLA

Bovine renal arterial endothelial cells (BRAEC) (Cell application Inc.), kindly provided by Dr. Kerry Sondgeroth, were grown and maintained according to the manufacturer's instructions. Briefly, BRAEC grown in a T-75 flask to 80% confluence were trypsinized for 1 min, neutralized, centrifuged at 220×*g* for 5 min, re-suspended in 5 ml of bovine endothelial cell growth medium, and counted with a hemocytometer under a phase contrast microscope. A total of 4000 cells per cm^2 were inoculated into a coated 48 well plate using 1 ml medium per well containing the respective TFLA at 10 µM. Identical cultures were also initiated using imidocarb dipropionate at 5 µM. Cultures without TFLA, and containing only the solvents at the highest concentration used in

TFLA (0.7%), were prepared as controls. The culture medium was replaced every day for a total of 72 h with 1 ml medium per well containing the respective drug. BRAEC cultured to 90% confluence after 72 h were trypsinized for 1 min, neutralized, centrifuged at 220×*g* for 5 min and resuspended in 0.4 ml of bovine endothelial cell growth medium. Ten microliters of resuspended BRAEC were transferred to a microcentrifuge tube containing 10 μl of 0.4% Trypan blue (Sigma) and the cells were immediately counted with a hemocytometer under an optical microscope. These experiments were carried out in triplicate.

2.9. Statistical analysis

The difference in the PPE for the *in vitro* culture with TFLA and the control (no drug added) were analyzed using independent Student's *t* test, where *p* values of <0.05 were considered statistically significant.

3. Results and discussion

3.1. The B. bovis α-tubulin amino acid sequence contains conserved consensus trifluralin binding sites

The full α-tubulin amino acid sequences of several parasites that are sensitive to trifluralin analogues were initially compared with the *B. bovis* α-tubulin amino acid sequence using the program ClustalW. The amino acid sequence comparisons showed high overall similarity among the *B. bovis* α-tubulin sequence and other protozoa α-tubulin sequences such as *L. major*, *P. falciparum*, *T. gondii*, *T. brucei gambiense*, *T. annulata*, and also with the *H. sapiens* α-tubulin sequence (Fig. 1). Consistent with the phylogenetic relationships among these organisms, the highest overall sequence identity found for the *B. bovis* sequence was for *T. annulata* (79.0%), followed by *T. gondii* (74.0%), *P. falciparum* (73.0%), *T. brucei gambiense* (70.0%), *L. major* (69.0%) and *H. sapiens* (65.0%) (Supplementary Fig. 1).

Previous studies demonstrated that certain amino acid residues of α-tubulin, labeled in gray font in Fig. 1, are critical for the binding of trifluralin (Traub-Cseko et al., 2001; Mitra and Sept, 2006; Hashim et al., 2012). Furthermore, it was found that certain amino acid mutations correlate with increased or decreased inhibition of the target cells growth by trifluralin. Importantly, most amino acid residues which are required for trifluralin binding (labeled in gray, Fig. 1), or that influence trifluralin sensitivity (amino acid in boxes, Fig. 1), are also conserved in the sequence of the *B. bovis* α-tubulin. However, the sequence comparison also shows that the *B. bovis* α-tubulin amino acid sequence differs in several residues that may be important for trifluralin binding, including Ser165Thr, Val171Ile, Leu230Ile, and Gly366Asp. These amino acid polymorphisms might potentially result in increased dinitroaniline-resistance in *B. bovis* when compared to the highly trifluralin sensitive wild type strain of *Leishmania* sp., similar to what was previously described for *T. gondii* trifluralin resistant mutants. Other amino acids that were found to be present only in *B. bovis* α-tubulin include: Cys6Ala, Cys20Val, Met36Val, Lys40Tyr, Cys41Gly, Ile42Ala, Asp47His, Val235Ile and Ala240Thr (Fig. 1). The N-terminal MREVI α-tubulin sequence which is involved in trifluralin binding (Chan et al., 1993; Hashim et al., 2012), is conserved among *P. falciparum*, *T. gondii* and *B. bovis*, but not in *L. major*, *T. brucei*, *H. sapiens* and *T. annulata*. Whether the overall changes found in the *B. bovis* α-tubulin amino acid sequence result in decreased or increased binding affinity by trifluralin or to novel TFLA compared to other protozoan parasites remains unknown, and thus we proceeded to test the ability of TFLA to inhibit the *in vitro* growth of cultured *B. bovis* parasites using the three newly synthetized and the previously tested TFLA 1–4 and 6–12 (henceforth referred as TFLA 1–12, although the TFLA 5 was not available for testing in this study). Although the definitive mechanisms of action of TFLA remain unknown, recent evidence suggests possible scenarios. In *P. falciparum* it was demonstrated that the dinitroanilines trifluralin and oryzalin inhibited growth of erythrocytic *P. falciparum* through schizogony, blocked mitotic division, and caused accumulation of abnormal microtubular structures (Fennell et al., 2006). It has also been demonstrated in *Toxoplasma* that when oryzalin binds to α-tubulin, under the N loop between protofilaments in the microtubule, it may then inhibit the N loop interaction with the M loop of the adjacent protofilament. This may result in instability of the M–N loop, followed by microtubule disruption (Snyder et al., 2001; Li et al., 2002; Morrissette et al., 2004). In addition, *Toxoplasma* resistance to a dinitroaniline derivate, oryzalin, is associated with point mutations in α-tubulin. Interestingly, most mutations leading to oryzalin resistance cluster in the core of the proteins, affecting the conformation of the α-tubulin dinitroaniline binding site (Morrissette et al., 2004).

3.2. Synthesis and chemistry of novel trifluralin analogues TFLA 13–15

The synthesis and characterization of TFLA 1 to TFLA 12 were reported previously (Esteves et al., 2010). Following the same general method, the synthesis of the new TFLA, namely TFLA 13–15, was accomplished by reacting the commercially available chlorinated precursor, chloralin, with imidazole, benzimidazole and sulfanilamide in the presence of triethylamine using ethanol as solvent (Fig. 2 and in Supplementary Fig. 2). The resulting new TFLA 13–15 were obtained in 60–70% yields, and are stable and easily-handled crystalline solids. The TFLA were fully characterized by FT-IR, MS, NMR and elemental analysis, and their water solubility was determined by UV spectrophotometry as described previously (Esteves et al., 2010). None of the TFLA 13–15 showed increases in their water solubility in comparison with TFLA 1–12, displaying low solubility parameters in all cases (<5 ppm).

3.3. Inhibition effect in in vitro B. bovis growth by TFLA 1–15

Because the use of the non-aqueous solvents needed to solubilize TFLA might be inhibitory for *B. bovis* parasites and/or toxic for the bovine erythrocytes, we first determined the effects of dimethyl sulfoxide (DMSO) and ethanol solvents on *B. bovis* in *in vitro* culture system, by direct observations of Giemsa stained cells under an optical microscope using 1000× amplification. Microscopic observations suggest that the addition of a mix of DMSO/ethanol at the concentration used for solubilizing the fourteen TFLA tested in this study (0.7% per well) into the *B. bovis* culture media does not result in apparent changes in the morphology of either the parasites or bovine erythrocytes (data not shown). However, the *in vitro B. bovis* culture grown in the presence of DMSO/ethanol showed a decrease in the growth rate of approximately 9.8% (±6.4) when compared with the *in vitro B. bovis* growth rate obtained using regular media (data not shown). These data are in accordance with previous results suggesting that this solvent can only slightly inhibit *in vitro* parasite growth and therefore it can be considered acceptable for culturing, when added at low concentrations into the culture media (Wadhwani et al., 2009).

We then investigated the efficacy of the TFLA on *B. bovis* parasites of the *in vitro* cultured biologically cloned strain Mo7 by performing the cultures in the presence of 10, 50 and 100 μM of TFLA 1–15. The cultures were examined every 24, 48 and 72 h for changes in PPE and the possible occurrence of morphology abnormalities by optical microscopy. Different levels of inhibition of the growth of the *B. bovis* Mo7 strain were observed for all 14 compounds tested when compared to cultures grown in the absence

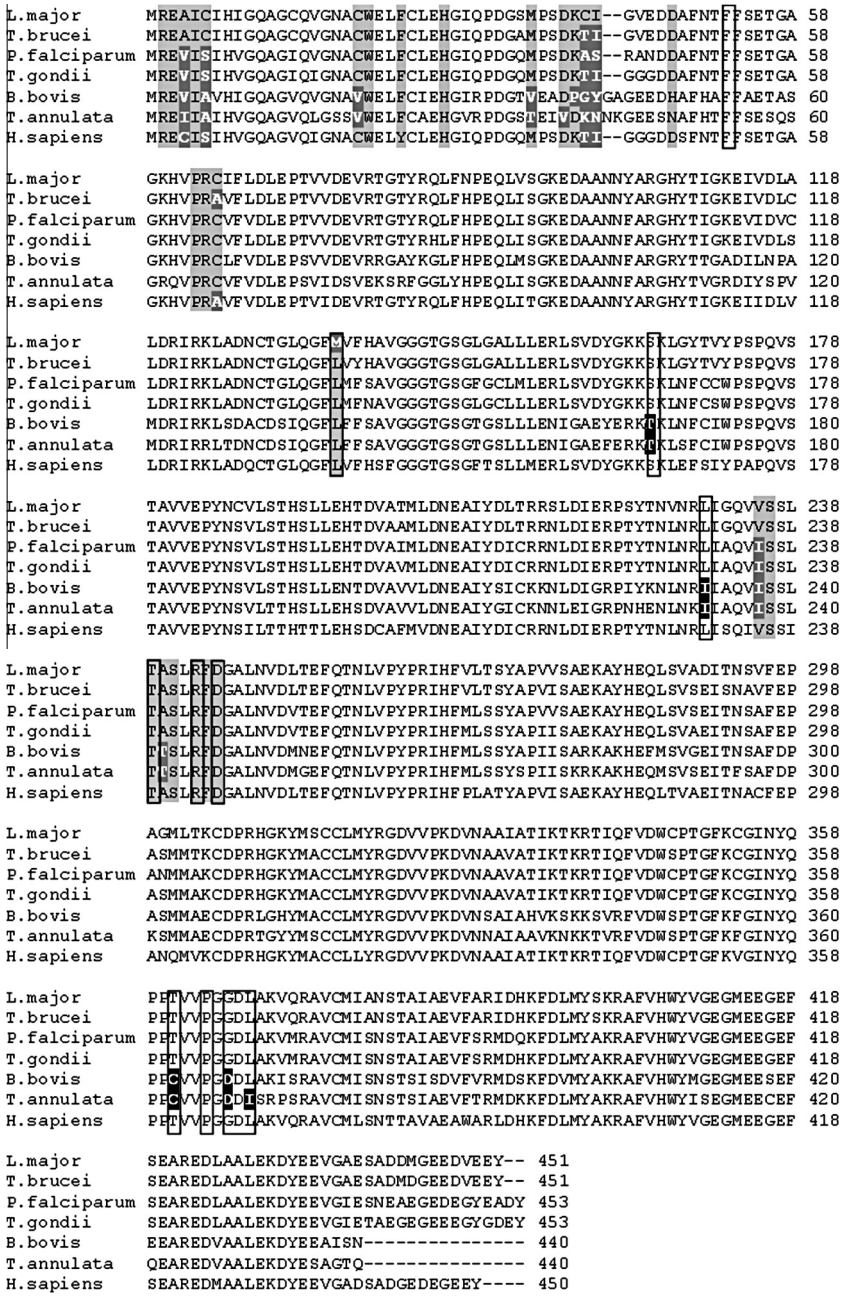

Fig. 1. CLUSTALW multiple alignment of deduced amino acid sequences of α-tubulin from protozoa parasites *Leishmania major* (*L. major*), *Trypanosoma brucei gambiense* (*T. brucei*), *Plasmodium falciparum* (*P. falciparum*), *Toxoplasma gondii* (*T. gondii*), *Babesia bovis* (*B. bovis*), *Theileria annulata* (*T. annulata*) and *Homo sapiens* (*H. sapiens*). The GenBank accession numbers are: CAJ02502.1; CBH08955.1; CAA34101.1; AAA30145.1; EDO06790.1; XP_954407.1 and NP_116093.1, respectively. The α-tubulin amino acids involved in the consensus binding site for dinitroaniline are highlighted in gray (Morrissette et al., 2004; Mitra and Sept, 2006; Hashim et al., 2012). Boxes indicate positions of amino acids that are not known to be involved in the binding site but might indirectly increase resistance to dinitroanilines, and white font indicates amino acids changes that are known to increase resistance to dinitroaniline (Traub-Cseko et al., 2001; Morrissette et al., 2004; Mitra and Sept, 2006; Ma et al., 2008; Hashim et al., 2012).

of the TFLA compounds. Their estimated 50% inhibitory concentration (IC_{50}) values were calculated (μM) and shown in Fig. 3A and in Supplementary Table 1. TFLA 7, TFLA 10 and the novel TFLA 13 were the compounds that showed the highest *B. bovis* Mo7 growth inhibition, with an estimated IC_{50} (μM) of 8.5 ± 0.3; 9.2 ± 0.2; 8.9 ± 0.7 at 72 h, respectively (Fig. 3B). Furthermore, the IC_{50} values for these three TFLA are statistically significant with p values <0.05. The three compounds with the highest inhibitory effect, TFLA 7, TFLA 10, and TFLA 13, were also tested for their ability to inhibit the growth of the virulent T_3Bo *B. bovis* strain in cultures. The estimated IC_{50} values for T_3Bo (μM) were 13.6 ± 1.5; 18.7 ± 1.6; 10.6 ± 1.9 at 72 h for TFLA 7, TFLA 10 and TFLA 13, respectively (Fig. 3B), in all cases consistent with the results obtained for the Mo7 strain. The statistically significant differences found among the IC_{50}'s of the Mo7 and T_3Bo parasites might be due to the clonal nature of the attenuated *B. bovis* Mo7 strain, in contrast to the polyclonal and heterogeneous composition of the virulent T_3Bo strain. No apparent morphological changes were detected among *B. bovis* parasites exposed to these compounds (data not shown). In *L. infantum*, the most effective compounds were TFLA 3, 8 and 10 (with IC_{50} (μM) of 1.2 ± 0.7; 1.1 ± 0.4, and 0.5 ± 0.1, respectively) with TFLA 7 having an IC_{50} (μM) of 2.2 ± 0.5 (Supplementary Ta-

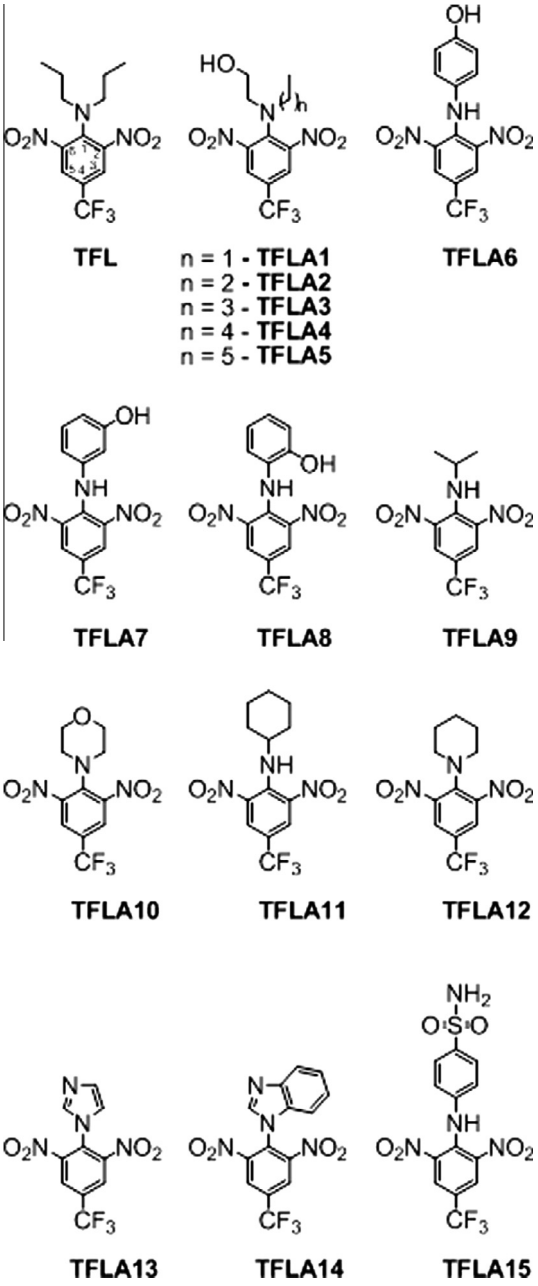

Fig. 2. Representation of the TFLA 1–TFLA 15 structures.

ble 2), (Esteves et al., 2010). However, overall comparison of the IC_{50} (μM) values between *B. bovis* and *L. infantum* (Supplementary Table 2) suggests that the numerous changes in α-tubulin binding amino acid residues among these molecules do not seem to have dramatic effects in the levels of sensitivity to trifluralin between these two parasites.

As a positive control we also performed parasite *in vitro* cultures in the presence of 5 μM of imidocarb dipropionate, which resulted in 100% inhibition of both *B. bovis* strains (data not shown). However, this drug produced visible morphological changes on both the T_3Bo and Mo7 *B. bovis* parasites as well as on erythrocytes, and drug concentrations that are equal or higher than 20 μM resulted in high levels of erythrocyte lysis as well (data not shown). Additionally, when imidocarb dipropionate was tested at its IC_{50} concentration (8.7×10^{-7} g ml^{-1}, or 1.75 μM; Rodriguez and Trees, 1996), caused 99.9% of inhibition of the Mo7 strain, producing similar visible morphological changes on erythrocytes and parasites as it was described above for the larger dose. The morphological changes observed on erythrocytes in imidocarb dipropionate-treated cultures include the presence of crenated erythrocytes, erythrocyte membrane ghosts, and irregular shaped parasites as described by Irwin, 2010. The morphology of most imidocarb dipropionate treated-parasites resemble crisis forms as previously described by Goff et al. (1998) and Herwaldt et al. (2004) (data not shown). TFLA 1 to TFLA 12, were also previously tested for their anti-leishmanial activity (Supplementary Fig. 2) (Esteves et al., 2010). TFLA 3 and TFLA 6–TFLA 11 were active against the intracellular form of the parasite and in particular the TFLA 3, TFLA 8, and TFLA 10, showed the highest significant reduction of the parasite load of the cells (Esteves et al., 2010). Although the establishment of a structure–activity relationship is beyond the objective of this work, we were able to identify some structural features that might potentially influence the anti-*B. bovis* activity of the drugs. For the analogues with a linear alkyl substituent in the amino group (TFLA 1 to TFLA 4), the results showed that there is an optimal length of the alkyl chain, specifically three carbons (TFLA 2). This trend was also observed in the anti-leishmanial activity of this series of compounds, but in this case the most active compound has a four carbon alkyl chain (TFLA 3) (Esteves et al., 2010). The two isomers, TFLA 6 and TFLA 7 showed a similar anti-*B. bovis* activity at 72 h, with TFLA 7 being the most active of all the compounds tested. However, the results showed a large decrease in the activity of the third isomer TFLA 8, which can be related to steric hindrance due to the proximity of the hydroxyl substituent and the amino group in the structure of this compound (Esteves et al., 2010). These proximities also favor the establishment of hydrogen bonds and might explain the higher water solubility of TFLA 8 when compared with TFLA 6 and TFLA 7. However, increase in water solubility is not correlated with increased activity, since TFLA 7 is the most potent inhibitory TFLA derivative tested in this study. An increase in the *B. bovis* inhibitory activity of these drugs was also observed when comparing results for TFLA 10 and TFLA 12, which is probably due to the introduction of an oxygen atom in the heterocyclic ring (TFLA 10). This effect was previously observed in the anti-leishmanial activity assays using TFLA (Esteves et al., 2010). Together, these observations might help future design of novel and improved anti-babesicidal TFLA.

3.4. Hemolytic activity of TFLA

We then determined the percentages of hemolysis of bovine erythrocytes as a surrogate marker of general membrane toxicity effects (Esteves et al., 2010), upon their exposure to TFLA 7, 10, and 13, the compounds that have the most potent inhibitory effect on both *B. bovis* strains tested (Fig. 4). The hemolytic activity of these TFLA was tested using a concentration of 10 μM, which is the approximate average IC_{50} value found among these three compounds. The data indicates that TFLA 13, which is the most effective anti-*B. bovis* compound, causes the less hemolysis (2%) followed by 3% for TFLA 10 and 4% for TFLA 7, respectively (Fig. 4). The percentages of hemolysis also augmented with increasing TFLA concentrations, reaching a maximum of approximately 11% for TFLA 7 and for TFLA 10 at 100 μM, and approximately 6% for TFLA 13 at an identical concentration. The percentage of hemolysis produced by the DMSO/ethanol solvent, at its highest concentration was of 2.5%. Interestingly, TFLA 7 and TFLA 10 showed similar curves of hemolysis throughout all the concentrations tested, from 10 to 100 μM.

Overall, and consistent with data previously reported by Esteves et al. (2010), these three TFLA display low hemolytic activity on bovine erythrocytes when exposed under a concentration of 20 μM, suggesting that they have a low level of toxicity for the bovine host

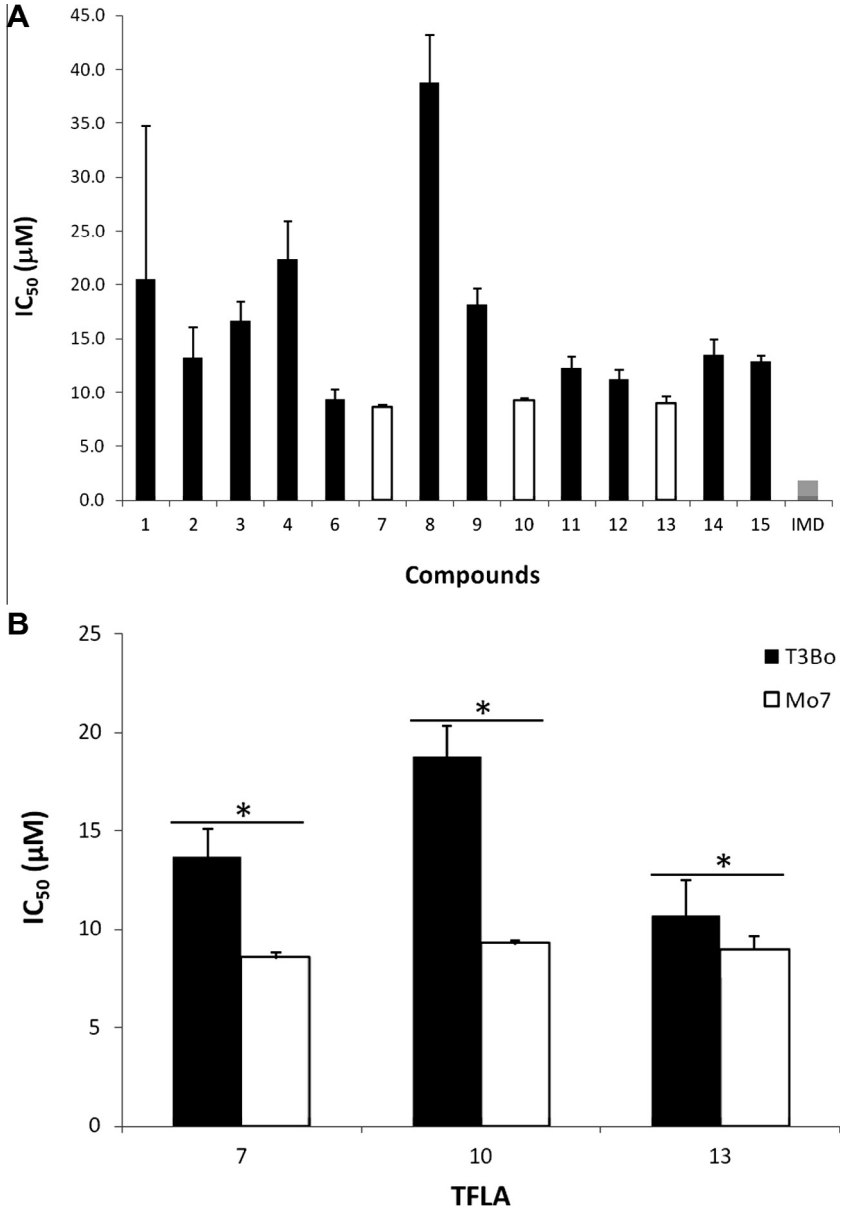

Fig. 3. IC_{50} (μM) values obtained from TFLA tested in *B. bovis* culture at 72 h after the addition of the TFLA as indicated in the *X* axis. (A) IC_{50} (μM) values from TFLA 1–4 and 6–15 obtained for the *B. bovis* Mo7 strain. The TFLA tested with lowest IC_{50} (TFLA 7, 10, and 13) are represented in white bars; imidocarb dipropionate (IMD) (Rodriguez and Trees, 1996) is represented in grey bar; (B) IC_{50} (μM) values for TFLA 7, 10 and 13 obtained for the *B. bovis* Mo7 strain (white bars) and T_3Bo strain (black bars). (∗) Represents *p*-value <0.05 indicating a statistically significant difference between strains. Both assays were carried out in triplicate. Error bars indicate standard error of the means for each TFLA tested.

cells. In contrast, the widely used imidocarb dipropionate exhibited much higher hemolytic activities, ranging from 9.5% to 15% of hemolysis for 5, 10 and 20 μM (Fig. 4). In addition, incubation of *B. bovis* cultures with imidocarb dipropionate at the IC_{50} concentration, (1.75 μM) resulted in a percentage of hemolysis of 6.6% (Fig. 4). The percentage of viability of these cultured parasites was 0.1% and *B. bovis* PPE of ∼0.1% at 72 h (Fig. 5). These data indicates that imidocarb dipropionate causes stronger hemolytic effects on bovine erythrocytes than the TFLA, even when used at the IC_{50} concentration.

3.5. Parasite and host cell viability after incubation with TFLA

We performed two experiments in order to determine the potential of the TFLA 7, 10 and 13 to affect the parasite and host cell viability after incubation with the drugs for 72 h. To test the effect of the TFLA on the viability of the parasite, TFLA-treated and non-treated control *B. bovis* infected erythrocytes were labeled with CFDA. Percentages of viable parasites were then determined by fluorescence microscopy for all treatments. Treatment with TFLA 7, TFLA 10, TFLA 13 and DMSO/ethanol resulted in percentages of parasite viability of 73, 93, 67 and 97, respectively (Fig. 5). The incubation of *B. bovis* infected erythrocytes with TFLA 7 and TFLA 13 resulted in a statistically significant decrease on the percentage of cell viability (*p* values <0.05), (Fig. 5).

To test the potential of the TFLA 7, 10 and 13 to affect viability of the bovine cell line BRAEC, we grew these cells in the presence of the TFLA at 10 μM followed by cell counting after the addition of Trypan blue. The data shows that neither TFLA 7, TFLA 10 nor the solvent ethanol/DMSO are able to significantly inhibit the growth

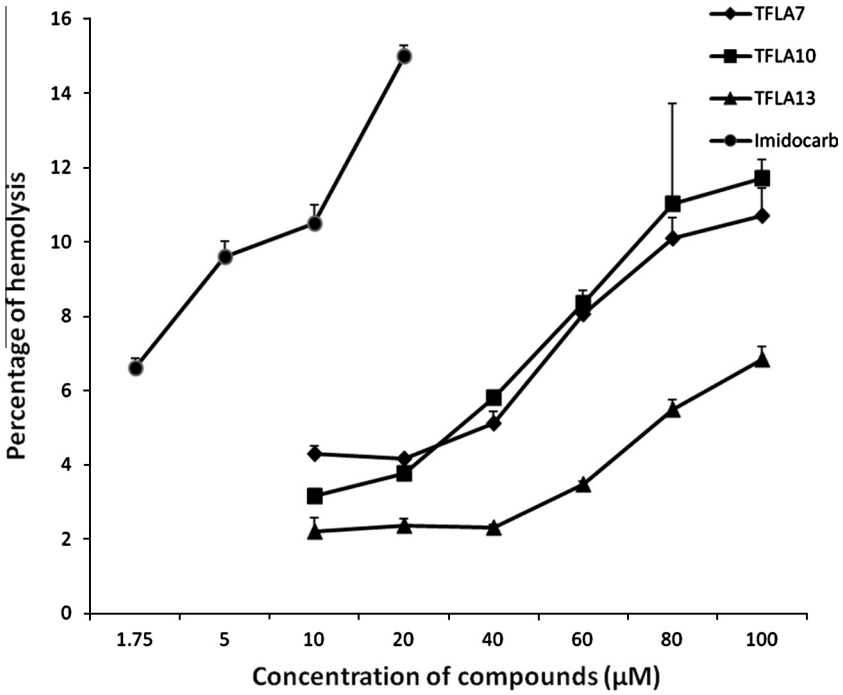

Fig. 4. Hemolytic activity calculated after erythrocytes were exposed to TFLA 7 (♦), TFLA 10 (■) and TFLA 13 (▲) at the following concentrations: 10, 20, 40, 60, 80 and 100 μM, and imidocarb dipropionate (•) used at 1.75, 5, 10 and 20 μM. The hemolytic activity was calculated by measuring the cells supernatant in a spectrophotometer at 550 nm compared with the reference filter at 620 nm. Absorbance of cell supernatant after bovine erythrocytes incubated with water was considered as 100% hemolysis. All assays were carried out in triplicate. Error bars indicate the standard error of the means for each TFLA tested.

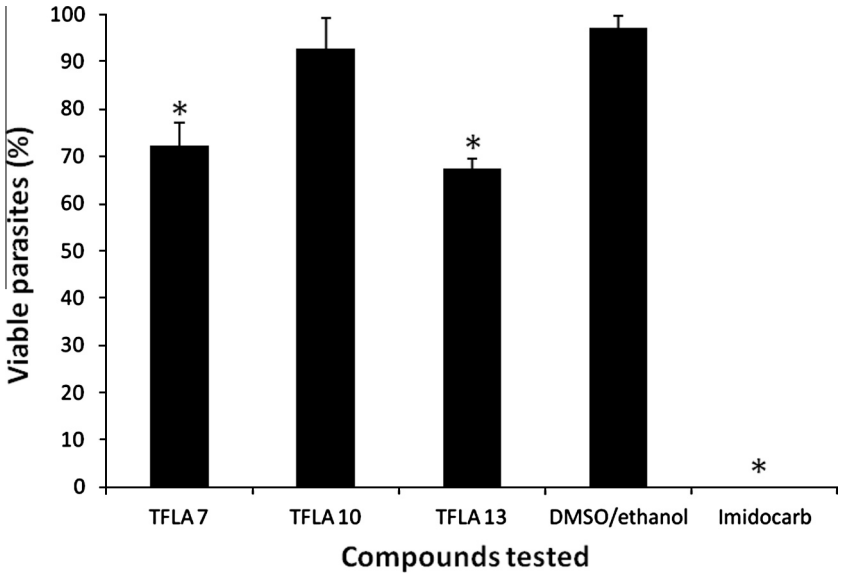

Fig. 5. Viability of *B. bovis* infected erythrocytes after incubation with TFLA 7, TFLA 10 and TFLA 13 at 10 μM, imidocarb dipropionate at 1.75 μM and with DMSO/ethanol at 72 h. *Y*-axis represents percentage of viable parasites [(parasites-labeled with 6-carboxyfluorescein diacetate/parasites-stained with Giemsa) ∗ 100]. The assays were carried out in triplicate. Error bars indicate standard error of the means for each TFLA tested. (∗) Represents *p*-value <0.05 indicating a statistically significant difference compared to the non-treated control.

of BRAEC, or to kill the cells (Fig. 6). In all cases, the results showed percentages of viability that are above 85% (Fig. 6), which is in accordance with the percentages of viability obtained when no drugs or additional solvents were applied (non-treated control) (Fig. 6). However, incubation of BRAEC with TFLA 13 resulted in a small but statistically significant decrease (∼20%) on the percentage of cell viability (*p* values <0.05), (Fig. 6).

Both set of experiments showed that addition of TFLA 7, TFLA 10 and TFLA 13 to *B. bovis in vitro* cultures resulted in significantly decreased levels of parasitemia compared to the control non-treated parasites. However most treated parasites remain viable, suggesting that TFLA might mainly act as cytostatic agents. In addition, TFLA 7 and TFLA 10 did not shown significant toxic effects for bovine cells. Overall, based on the viability and toxicity results, the

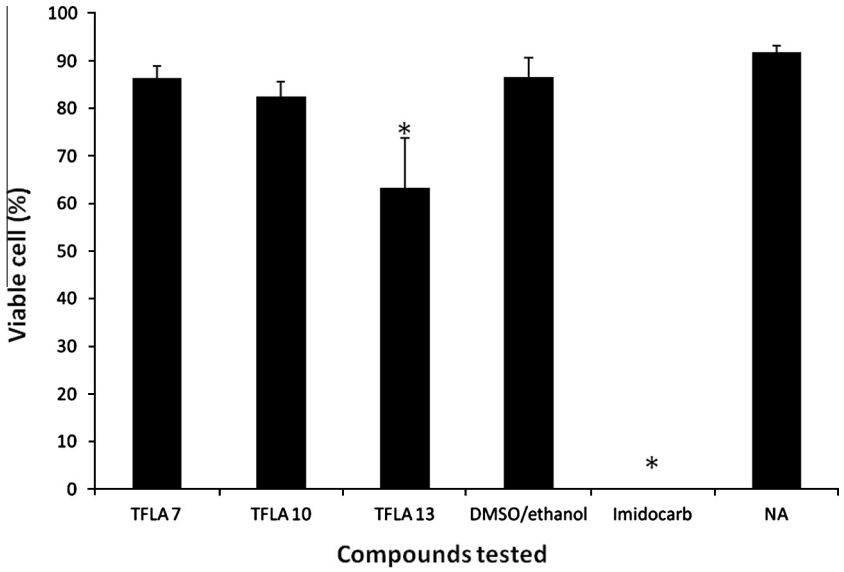

Fig. 6. Viability of BRAEC after incubation with TFLA 7, TFLA 10 and TFLA 13 at 10 μM, DMSO/ethanol and non-treated well (without the addition of drugs, represented as NA) at 72 h using Trypan blue. The assays were carried out in triplicate. Error bars indicate standard error of the means for each TFLA tested. (*) Represents p-value <0.05 indicating a statistically significant difference compared to the non-treated control.

data suggest that TFLA 7 and TFLA 10 may be further developed as novel alternatives for the treatment of bovine babesiosis.

4. Conclusions

TFLA are able to effectively inhibit the *in vitro* growth of *B. bovis* without significantly compromising the host erythrocyte cells. The TFLA 7, 10 and 13 were found to be the most potent inhibitors of *B. bovis* in *in vitro* cultures, likely acting using a cytostatic mechanism. Neither TFLA 7 nor TFLA 10 exerted significant toxic effects on a bovine cell line, although a small but significant effect on these cells was found for TFLA 13. No dramatic differences in the IC_{50}'s were found between *Leishmania* sp. and *B. bovis* despite several changes in the amino acid sequences that are known to be critical for the binding of α-tubulin to trifluralin. Furthermore, this explanation can be also extended to the trifluralin-sensitivity differences found among *B. bovis*, *Leishmania* sp., *T. gondii* and *P. falciparum*. However, and because we tested other distinct and novel TFLA in this study, we are not able to exclude differential sensitivity due to chemistry differences, and/or differences in drug uptake, metabolism, export or processing, or other biological features, in each parasite species. However, experimental evidence suggests that increased water solubility of the compounds does not necessarily translate into augmented anti-babesicidal activity. Thus, the differences in sensitivity found can also be reflective of the distinct parasite intracellular lifestyles and their intracellular milieus among other possible factors.

Further studies, such as docking analysis, would improve our understanding of the observed differences and guide rational babesicidal drug designs. Further research aimed at determining the effect of TFLA *in vivo* may lead to the development of improved and more effective methods to control bovine babesiosis using pharmacological approaches.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpddr.2013.01.003.

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